

FORM-PTO-1390  
(Rev. 12-29-99)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

001560-393

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)

09/787737

INTERNATIONAL APPLICATION NO.

PCT/JP00/04904

INTERNATIONAL FILING DATE

21 July 2000

PRIORITY DATE CLAIMED

22 July 1999

TITLE OF INVENTION

HOMOEBOX GENE ENCODING A PROTEIN INVOLVED IN DIFFERENTIATION

APPLICANT(S) FOR DO/EO/US

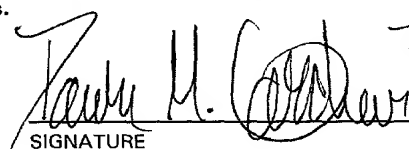
Tatsuo KAKIMOTO

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
- ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
- ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.  
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:  
Copy of Japanese PCT Request Form  
Copy of cover page from published PCT international application (WO 01/07618)  
Notice Informing Applicant of Communication of the International Application to the Designated Offices  
International Search Report

U.S. APPLICATION NO. (If known, see 37 CFR 1.101) <b>09/787737</b>		INTERNATIONAL APPLICATION NO. PCT/JP00/04904		ATTORNEY'S DOCKET NUMBER 001560-393	
17. <input type="checkbox"/> The following fees are submitted:				CALCULATIONS	PTO USE ONLY
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b>  Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1,000.00 (960)  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00 (970)  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00 (958)  International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00 (956)  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00 (962)					
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				\$ 860.00	
Surcharge of \$130.00 (154) for furnishing the oath or declaration later than 20 <input type="checkbox"/> 30 <input type="checkbox"/> months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	27 -20 =	7	X\$18.00 (966)	\$ 136.00	
Independent Claims	6 -3 =	3	X\$80.00 (964)	\$ 240.00	
Multiple dependent claim(s) (if applicable)				+ \$270.00 (968)	\$
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$ 1,236.00	
Reduction for 1/2 for filing by small entity, if applicable (see below).				\$	-
<b>SUBTOTAL =</b>				\$ 1,236.00	
Processing fee of \$130.00 (156) for furnishing the English translation later than 20 <input type="checkbox"/> 30 <input type="checkbox"/> months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
<b>TOTAL NATIONAL FEE =</b>				\$ 1,236.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 (581) per property +				\$ 40.00	
<b>TOTAL FEES ENCLOSED =</b>				\$ 1,276.00	
				Amount to be:	
				refunded	\$
				charged	\$
a. <input type="checkbox"/> Small entity status is hereby claimed. b. <input checked="" type="checkbox"/> A check in the amount of \$ 1,276.00 to cover the above fees is enclosed. c. <input type="checkbox"/> Please charge my Deposit Account No. 02-4800 in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed. d. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.					
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>					
SEND ALL CORRESPONDENCE TO:  Ronald L. Grudziecki BURNS, DOANE, SWECKER & MATHIS, L.L.P. P.O. Box 1404 Alexandria, Virginia 22313-1404 (703) 836-6620					
				 SIGNATURE	
				Donna M. Meuth NAME	
				36,607 REGISTRATION NUMBER	

09/787737

JC10 Rec'd PCT/PTO 2 2 MAR 2001  
Patent

Attorney's Docket No. 001560-393

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of )  
)  
Tatsuo KAKIMOTO ) Group Art Unit: Unassigned  
)  
Application No.: TBA (§371 of ) Examiner: Unassigned  
PCT/JP00/04904) )  
)  
Filed: Even date herewith )  
)  
For: HOMEBOX GENE ENCODING A )  
PROTEIN INVOLVED IN )  
DIFFERENTIATION )

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application on the merits, please amend  
the application as follows:

**IN THE SPECIFICATION:**

Please add the paper copy of the Sequence Listing included herewith to the  
application, after page 17 and before the Claims on page 18.

Please renumber the pages accordingly.

09/787737 "032301"

IN THE CLAIMS:

Please replace claims 7-9, 11, 15, 16-18 as follows:

7. (Amended) The gene according to claim 1 wherein said protein is a protein having an ability of inducing adventitious shoots.

8. (Amended) The gene according to claim 1 wherein said protein is a protein having an ability of inducing branching.

9. (Amended) A vector comprising the gene according to claim 1.

11. (Amended) A protein encoded by the gene according to claim 1.

15. (Amended) A plant or a plant cell into which the gene according to claim 1 has been introduced.

16. (Amended) A method for inducing differentiation from a plant or a plant cell said method comprising introducing the gene according to claim 1 into a plant or a plant cell and then driving the expression of said gene.

17. (Amended) A method for inducing adventitious shoot formation from a plant or a plant cell said method comprising introducing the gene according to claim 1 into a plant or a plant cell and then driving the expression of said gene.

18. (Amended) A method for inducing branching of a plant said method comprising introducing the gene according to claim 1 into a plant and then driving the expression of said gene.

Please add new claims 19-27 as follows:

--19. The gene according to claim 2 wherein said protein is a protein having an ability of inducing adventitious shoots.

20. The gene according to claim 2 wherein said protein is a protein having an ability of inducing branching.

21. A vector comprising the gene according to claim 2.

22. A host transformed with the vector according to claim 21.

23. A protein encoded by the gene according to claim 2.

24. A plant or a plant cell into which the gene according to claim 2 has been introduced.

25. A method for inducing differentiation from a plant or a plant cell said method comprising introducing the gene according to claim 2 into a plant or a plant cell and then driving the expression of said gene.

26. A method for inducing adventitious shoot formation from a plant or a plant cell said method comprising introducing the gene according to claim 2 into a plant or a plant cell and then driving the expression of said gene.

27. A method for inducing branching of a plant said method comprising introducing the gene according to claim 2 into a plant and then driving the expression of said gene.--

**REMARKS**

Prior to examination, entry of the foregoing is respectfully requested.

Claims 7-9, 11, 15, 16-18 have been amended simply to delete multiple dependencies in the claims. New claims 19-27 have been added, directed to embodiments dependent upon claim 2 in view of deletion of the multiple dependencies. Support for the new claims may be found in original claims 7-9, 11, 15, 16-18. No new matter has been added.

In the event that there are any questions relating to this Preliminary Amendment, or to the application in general, it would be appreciated if the Examiner would telephone the undersigned attorney at (508) 339-3684 concerning such questions so that prosecution of this application may be expedited.

Early and favorable action in the form of a Notice of Allowance is respectfully requested and believed to be in order.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: 

Donna M. Meuth

#66 Registration No. 36,607

Reg 100  
2/24/18

P.O. Box 1404  
Alexandria, Virginia 22313-1404  
(703) 836-6620

Date: March 22, 2001

**Attachment to Preliminary Amendment dated March 22, 2001**

**Marked-up Claims 7-9, 11, 15, 16-18**

7. (Amended) The gene according to [any one of the claims 1 to 6] claim 1 wherein said protein is a protein having an ability of inducing adventitious shoots.
8. (Amended) The gene according to [any one of the claims 1 to 6] claim 1 wherein said protein is a protein having an ability of inducing branching.
9. (Amended) A vector comprising the gene according to [any one of the claims 1 to 8] claim 1.
11. (Amended) A protein encoded by the gene according to [any one of the claims 1 to 8] claim 1.
15. (Amended) A plant or a plant cell into which the gene according to [any one of the claims 1 to 8] claim 1 has been introduced.
16. (Amended) A method for inducing differentiation from a plant or a plant cell said method comprising introducing the gene according to [any one of the claims 1 to 8] claim 1 into a plant or a plant cell and then driving the expression of said gene.
17. (Amended) A method for inducing adventitious shoot formation from a plant or a plant cell said method comprising introducing the gene according to [any one of the claims 1 to 8] claim 1 into a plant or a plant cell and then driving the expression of said gene.
18. (Amended) A method for inducing branching of a plant said method comprising introducing the gene according to [any one of the claims 1 to 8] claim 1 into a plant and then driving the expression of said gene.

JC10 Rec'd PCT/PTO 2 2 MAR 2001

## DESCRIPTION

HOMEBOX GENE ENCODING A PROTEIN INVOLVED IN  
DIFFERENTIATION

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## Technical Field

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10 The present invention relates to gene encoding a  
protein that is involved in differentiation and that has  
a homeodomain-like sequence. More specifically, the  
present invention relates to genes encoding a protein  
that has an ability of inducing adventitious shoots and  
branching and that has a homeodomain-like sequence, and  
uses thereof.

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## Background Art

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20 Plants generally have totipotency and, for example,  
can regenerate individual plants through the regeneration  
of adventitious shoots or adventitious embryos from  
undifferentiated tissues derived from somatic cells.  
21 This ability is used for, for example, the production of  
young plants by cultured shoot. In addition, the  
technique of regenerating transformed plants via the  
regeneration of adventitious shoots or adventitious  
embryos after the introduction of genes into plant  
22 somatic cell tissues or cultured plant cells has become  
an indispensable technology in the field of plant  
biotechnology in recent years. It is generally thought  
that the regeneration of adventitious roots or  
adventitious shoots from callus, which is an  
23 undifferentiated cells or plant tissues originated from  
leaves, stems, and the like is regulated by the  
interaction of plant hormones such as auxins and  
cytokinins.

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34 For plant morphogenesis, it has also been reported  
that a series of genes including homeobox are involved in  
addition to plant hormones. Homeobox genes was found as  
a well-conserved 183 bp DNA sequence occurring in common



in certain genes that regulate the development of  
Drosophila. The 61 amino acid sequence translated from  
this region is called homeodomain, which takes a helix-  
turn-helix structure comprising three  $\alpha$  helices and  
5 which recognizes a specific nucleotide sequence thereby  
to bind DNA.

Animal homeobox genes have been elucidated to be  
transcription factors that control development processes,  
whereas for plants the KNOTTED1 (KN1) gene isolated from  
10 corn in 1991 is the first homeobox gene in higher plants  
(Vollbrecht et al., Nature 350: 241-243, 1991). Although  
veins of corn leaves are parallel ones, Knotted1 mutation  
results in disturbances in veins and drives formation of  
knot-like processes along veins, after which it was named  
15 Knotted.

On the other hand, using synthetic DNA corresponding  
to specifically highly conserved amino acid sequences in  
the homeobox that had been found in many animals, genomic  
DNA of a dicotyledon *Arabidopsis thaliana* was searched  
20 with a result that several homeobox genes were reported  
(Ruberti et al., EMBO J. 10: 1787-1791, 1991).

Homeobox genes of higher plants reported so far have  
been roughly grouped into five types based on the  
similarity of amino acid composition in the homeodomain  
25 and the structure of regions other than the homeobox  
domain (Tasaka, Tanpakushitu Kakusan Koso (Proteins,  
Nucleic Acids, Enzymes) 40(8): 1033-1042, 1995). The  
first type is represented by the KN1 gene of corn, the  
second type has the homeobox approximately in the center  
30 of a protein, of which a C-terminal end is flanked by a  
regularly repeated structure of leucine moieties (leucine  
zipper) that are involved in dimer formation of the  
protein. The third type has the homeodomain near the C-  
terminal end of a protein, and a finger structure of the  
35 metal-bound type at the N-terminal end. The fourth type,  
in addition to having a structure common to the third  
type, has repeated structures of several amino acid

sequences. The fifth type has the homeobox in the N-terminal end of a protein and no other well-known characteristic structures have been found therein.

5 The overall homology of amino acid sequences between  
the different types is 32 to 58% in the homeodomain.  
However, as can be estimated from a report that the third  
helix in the homeodomain enters into the main groove of  
the target double stranded DNA to control transcription  
when a protein containing an animal homeodomain binds to  
10 DNA, this third helix has the highest homology  
irrespective of the type even in the gene products of  
plant homeoboxes. The region is thought to be essential  
for a homeodomain protein to bind to DNA as a  
transcription factor. Recently, a homeobox gene WUSCHEL  
15 was reported that does not belong to any of these five  
groups (Cell, 95: 805-815, 1998). Although the mutants  
defective in the function of the WUSCHEL gene cannot  
drive normal growth of apical meristem of the stem, there  
are no experimental reports on overexpression of the  
20 WUSCHEL gene, and it is unknown what changes may occur  
when the WUSCHEL gene expression is artificially  
enhanced.

The homeobox genes of plants have been suggested to  
be possibly involved in the control of organogenesis or  
25 development processes, infection protection, and  
regulation of material transport in the plants, details  
of which are not known, however. Protein having a  
homeobox is generally thought to serve as transcription  
factor, but even the target gene whose transcription is  
30 regulated by each homeodomain protein has not been  
elucidated. Furthermore, although the overexpression of  
the KN1 type among the homeobox genes causes extremely  
abnormal morphology, it is not known whether adventitious  
shoots are formed on the callus.

35 From the standpoint of agricultural application, a  
gene having a high ability of inducing adventitious  
shoots and branching on a cultured tissue such as callus

in the tissue culture system would be considered to be useful, but there are no such genes.

#### Disclosure of the Invention

5           Thus, it is an object of the present invention to provide a gene encoding a protein that is involved in differentiation and that has a homeodomain-like sequence, specifically a protein that has an ability of inducing adventitious shoots and branching, a protein encoded  
10           thereby, and uses thereof.

          The inventors of the present invention conducted activation tagging using *Arabidopsis thaliana* and have obtained a gene encoding a protein that has an ability of inducing adventitious shoots and branching. The  
15           activation tagging as used herein means a method of inserting enhancer sequences at random into a plant genome to isolate mutants in which the transcription of genes downstream of the inserted enhancer has been activated.

20           Thus, the present invention provides a gene encoding a protein that is involved in differentiation and that has a homeodomain-like sequence. More specifically, the present invention provides a gene encoding a protein that has an ability of inducing adventitious shoots and  
25           branching, and that has a homeodomain-like sequence.

          More specifically, the present invention provides a gene encoding a protein that has the amino acid sequence as set forth in SEQ ID NO: 2, that is involved in differentiation, and that has a homeodomain-like  
30           sequence. The present invention further provides a gene encoding a protein that has an amino acid sequence modified by the addition or deletion of one or a plurality of amino acids and/or replacement with other amino acids in the amino acid sequence as set forth in  
35           SEQ ID NO: 2, that is involved in differentiation, and that has a homeodomain-like sequence. The present invention further provides a gene that hybridizes to the

nucleic acid as set forth in SEQ ID NO: 1, specifically its DNA or a portion thereof, and that encodes a protein that is involved in differentiation and that has a homeodomain-like sequence.

5           The present invention further provides a gene encoding a protein that has the amino acid sequence as set forth in SEQ ID NO: 4, that is involved in differentiation, and that has a homeodomain-like sequence. The present invention further provides a gene  
10 encoding a protein that has an amino acid sequence modified by the addition or deletion of one or a plurality of amino acids and/or replacement with other amino acids in the amino acid sequence as set forth in SEQ ID NO: 4, that is involved in differentiation, and  
15 that has a homeodomain-like sequence. The present invention further provides a gene that hybridizes to the nucleic acid as set forth in SEQ ID NO: 3, specifically its DNA or a portion thereof, and that encodes a protein that is involved in differentiation, and that has a  
20 homeodomain-like sequence.

As used herein, a protein that is involved in differentiation and that has a homeodomain-like sequence is a protein that is involved in the process in which cells differentiate into morphologically and/or  
25 functionally different cells such as differentiation into adventitious shoots, branches, leaves, flowers or the like, and that has a homeodomain-like sequence functioning as a DNA-binding domain, and specifically a protein that induces the formation of adventitious  
30 shoots, a protein that induces branching, and the like.

The present invention also provides vectors comprising the gene.

35           The present invention further provides hosts transformed with the vector. The hosts may be plant cells or plants.

The present invention also provides a method for producing a protein that is involved in differentiation

and that has a homeodomain-like sequence by culturing and/or cultivating the above host.

5 The present invention also provides a method for inducing differentiation of plants or plant cells, said method comprising introducing the above gene into plants or plant cells and driving the expression of said gene.

10 The present invention also provides a method for inducing the formation of adventitious shoots of plants or plant cells, said method comprising introducing the above gene into plants or plant cells and driving the expression of said gene.

15 The present invention also provides a method for inducing branch formation of plants said method comprising introducing the above gene into plants and driving the expression of said gene.

#### Embodiment for Carrying Out the Invention

20 The inventors of the present invention investigated the possibility of identifying a gene involved in differentiation that leads to the induction of adventitious shoot formation on a callus when overexpressed by activation tagging. Thus, *Arabidopsis thaliana*-transformed calluses into which an activation tagging vector pPCVICEn4HPT had been introduced via  
25 *Agrobacterium* were screened on a cytokinin-free medium to isolate a transformant that formed adventitious shoots even in the absence of cytokinin (an adventitious shoot is not usually formed in the absence of cytokinin). Among them, a transformant designated "many shoot" (msh)  
30 formed adventitious shoots in the absence of cytokinin.

35 When the MSH gene that caused the phenotype of the msh mutant and the corresponding MSH cDNA were isolated and analyzed, it was found that the protein encoded by the MSH gene has an amino acid sequence having a significant homology with homeodomain, and, among others, the third  $\alpha$  helix domain of the homeodomain conserved in a series of homeodomain proteins had a high homology. In

addition, when the coding region of MSH cDNA was introduced into an *Arabidopsis thaliana* callus and was overexpressed, as can be indicated from the phenotype of the msh mutant, the transformed callus formed an adventitious shoot in the presence or absence of cytokinin in the medium. It was also found that in *Arabidopsis thaliana* transformants in which MSH cDNA was overexpressed, branching was more frequent than in the wild type *Arabidopsis thaliana*, and adventitious shoots were occasionally formed on the leaves.

The foregoing revealed that the MSH gene is involved in differentiation and encodes a protein having a homeodomain-like sequence, which indicated the possibility that the overexpression of this might result in enhanced ability of forming adventitious shoots and branching.

As the gene of the present invention, there can be mentioned one that encodes the amino acid sequence as set forth in SEQ ID NO: 2 or 4. However, it is known that a protein that has an amino acid sequence modified by the addition or deletion of one or a plurality of amino acids and/or replacement with other amino acids retain similar effects to the native protein. Thus, the present invention encompasses a protein that has an amino acid sequence modified by the addition or deletion of one or a plurality of amino acids and/or replacement with other amino acids in the amino acid sequence as set forth in SEQ ID NO: 2 or 4, and a gene encoding said protein.

As used herein, the degree of modification is one that is possible by means known prior to the filing of the present application such as site-directed mutagenesis and PCR. The number of amino acids targeted for modification while maintaining the ability of inducing adventitious shoots and branching is for example 50 or less, preferably 25 or less, for example 10 or less.

The present invention also provides a gene that hybridizes to the nucleic acid as set forth in SEQ ID NO:

1 or 3, specifically its DNA or a portion thereof under a stringent condition, and that encodes a protein that is involved in differentiation and that has a homeodomain-like sequence. The stringent condition as used herein  
5 means a condition in which hybridization occurs, for example, 5 x SSC and 50°C. A suitable hybridization temperature may be selected as appropriate since it varies with the nucleotide sequence or the length of the nucleotide sequence.

10 The above portion of a nucleic acid is a portion that encodes a sequence comprising at least several contiguous amino acids, and preferably a portion that encodes a sequence comprising at least several contiguous amino acids in the homeodomain. More preferably, it  
15 means a portion or a fragment that contains a part or all of the homeodomain sequence in the sequence as set forth in SEQ ID NO: 1 or 3, and that has a length of 25% or greater, for example 50% or greater, and more preferably 75% or greater of the entire sequence as set forth in SEQ  
20 ID NO: 1 or 3.

As a source of the gene as a target for the above hybridization, there can be used a cDNA library, a genomic DNA library, etc. prepared from plants, microorganisms etc., and as the plant, there can be  
25 mentioned *Arabidopsis thaliana*, petunia, snapdragons, rice, corn, tobacco, poplar, and the like.

The nucleotide sequence of the thus obtained gene encoding a protein that is involved in differentiation and that has a homeodomain-like sequence has a homology  
30 of 50% or greater, 60% or greater, preferably 70% or greater or 80% or greater, for example 90% or greater with the nucleotide sequence as set forth in SEQ ID NO: 1 or 3.

The gene of the present invention encoding a protein  
35 having the amino acid sequence as set forth in SEQ ID NO: 2 or 4 may be obtained from *Arabidopsis thaliana* as cDNA or genomic DNA.

As specifically shown in the examples, genes having the native nucleotide sequence can be obtained by, for example, screening cDNA libraries. DNA encoding a protein having a modified amino acid sequence can also be synthesized based on the DNA having the native nucleotide sequence by conventionally used site-directed mutagenesis or a PCR method. For example, a DNA fragment to be modified may be obtained by treating the native cDNA or genomic DNA with restriction enzymes, and using this as a template, site-directed mutagenesis or a PCR method is carried out using a primer into which the desired mutation has been introduced so as to obtain a DNA fragment into which the desired modification has been introduced. Then the mutation-introduced DNA fragment may be linked to a DNA fragment encoding another part of the protein of interest.

Alternatively, in order to obtain a DNA encoding a protein comprising a shortened amino acid sequence, a DNA encoding an amino acid sequence longer than the amino acid sequence of interest, for example a full-length amino acid sequence, is cleaved by a desired restriction enzyme, and when the resulting DNA fragment was found not to encode the entire amino acid sequence of interest, a DNA fragment comprising the lacking sequence is synthesized and ligated thereto.

By expressing the obtained gene using a gene expression system in *Escherichia coli* or yeast, the gene product MSH protein may be obtained. Alternatively, the MSH protein may be obtained by using an antibody against a protein encoded by the amino acid sequence as set forth in SEQ ID NO: 2 or 4. By using an antibody, the gene of a protein having a similar function to MSH may be cloned from another organism.

Thus, the present invention also relates to a recombinant vector comprising the above-mentioned gene, specifically an expression vector, and a host transformed with said vector. As a host, there can be used a



prokaryotic or eukaryotic organism. As a prokaryotic organism, there can be used such a common host as a microorganism belonging to the genus *Escherichia* such as *Escherichia coli*, a microorganism belonging to the genus *Bacillus* such as *Bacillus subtilis*, and the like.

As an eukaryotic host, there can be used a lower eukaryotic organism, for example an eukaryotic microorganism, for example a fungus, yeast or a mold. As yeast, there can be mentioned a microorganism belonging to the genus *Saccharomyces* such as *Saccharomyces cerevisiae*, and as a mold, there can be mentioned a microorganism belonging to the genus *Aspergillus* such as *Aspergillus oryzae* and *Aspergillus niger*, and a microorganism belonging to the genus *Penicillium*.

Furthermore, animal cells or plant cells can be used: as animal cells, there can be used cell lines derived from mouse, hamster, monkey, human and the like, specifically COS cells, Vero cells, CHO cells, L cells, C127 cells, BALB/c3T3 cells, Sp-2/0 cells, and the like. As plant cells, there can be used cultured cells from tabacco, genus *Populus*, genus *Eucalyptus*, genus *Acacia*, and the like.

Insect cells such as silkworm cells or adult silkworms per se can also be used as hosts.

Specifically, insect cells such as cells of *Spodoptera frugiperda*, cells of *Bombyx mori*, etc. may be used.

As vectors, there can be used plasmid, phage, phagemid, virus (baculovirus (insect cell expression system), vaccinia virus (animal cell expression system)) and the like.

The vectors of the present invention may contain expression regulatory regions such as a promoter, a terminator, an origin of replication, and the like, depending on the host into which said vector is to be introduced. As promoters for bacterial expression vectors, there can be used commonly used promoters such as trc promoter, tac promoter, lac promoter, and the

like; as promoters for yeasts, there can be used  
glyceraldehyde-3-phosphate dehydrogenase promoter, PH05  
promoter, adhI promoter, ptk promoter and the like; and  
as mold promoters, there can be used amylase promoter,  
5 trpC promoter, and the like.

As promoters for insects, there can be mentioned the  
baculovirus polyhedrin promoter etc.; as animal cells,  
there can be mentioned the early and late promoter of  
Simian Virus 40, CVM promoter, HSV-TK promoter or SR $\alpha$   
10 promoter, and the like.

Furthermore, as promoters for plants, there can be  
mentioned CaMV35S promoter, nopaline synthase promoter;  
as inducible promoters, there can be mentioned a promoter  
of the glutathione S-transferase II system genes, hsp80  
15 promoter, the promoter of ribulose-2-phosphate  
carboxylase small subunit gene, and the like.

Furthermore, preferred expression vectors may contain, in  
addition to the above, an enhancer, a splicing signal,  
polyA addition signal, a selectable marker such as  
20 dihydrofolate reductase gene (methotrexate-resistant) and  
neo gene (G418-resistant), and the like. When an  
enhancer is contained, the enhancer of SV40, for example,  
may be inserted into upstream or downstream of the gene.

The transformation of the host with an expression  
25 vector may be conducted according to a conventional  
method well known to a person skilled in the art, which  
is described in, for example, Current Protocols in  
Molecular Biology, John Wiley & Sons, 1995. The  
culturing of transformants can also be conducted  
30 according to a conventional method. Purification of a  
protein from the culture may be conducted by, for  
example, gel filtration, various column chromatography  
such as one that employs Sepharose, and the like. When  
it is expressed as a fusion protein with GST or  
35 polyhistidine in the host, it can be easily purified by  
suitable affinity chromatography.

Given the current state of the art, the promotion of

differentiation such as adventitious shoot formation or branching formation in plants such as roses for which plant regeneration is difficult even by artificial control such as the use of plant hormones can be attained by ligating cDNA or genomic DNA under the control of a constitutive or an inducible promoter, introducing the gene into a plant in a system that employs *Agrobacterium*, particle gun, or electroporation, and driving the expression thereof.

Furthermore, it will be possible to change the morphology of garden plants, for example changing a standard type into a spray type, by regulating the expression of the gene of the present invention and, as a result, to increase the number of flowers and leaves.

#### Examples

The present invention will now be explained in further details with reference to the following Examples. Molecular biological procedures were conducted according to Molecular Cloning (Sambrook et al., 1989) unless otherwise specified.

#### Example 1. Screening of a cytokinin responsive mutant

In order to obtain a mutant that exhibits cytokinin response even in the absence of cytokinin by increasing the amount of the transcribed gene involved in differentiation such as adventitious shoot formation and branching formation, activation tagging was conducted using *Arabidopsis thaliana*.

About 50,000 calluses of *Arabidopsis thaliana* were transformed with a vector pPCVICEn4HPT (Hayashi et al., Science, 258: 1350-1353, 1992) for activation tagging according to the method of Akama et al. (Akama et al., Plant Cell Rep., 12: 7, 1992). Since pPCVICEn4HPT has a strong enhancer sequence derived from the CaMV35S promoter, the transcription of gene adjacent to the enhancer sequence is activated after its insertion into the plant genome. After transformation, the transformed

calluses were cultured in a cytokinin-free medium.

Although the cellular growth of the wild type (non-transformed) *Arabidopsis thaliana* callus is suppressed and adventitious shoots cannot be formed in the cytokinin-free medium, some of the transformed calluses formed adventitious shoots even in the absence of cytokinin. Of them, a transformant that has a high ability of forming adventitious shoots and that forms many adventitious shoots was designated the msh (many shoot) transformant. When seeds obtained from the msh mutant were sown on a conventional agar medium for culturing *Arabidopsis thaliana*, many adventitious shoots were observed on the cotyledons.

Example 2. Isolation of the causative gene MSH of the msh mutant

Genomic DNA was extracted from the msh mutant obtained in Example 1. After this genomic DNA was treated with a restriction enzyme *SacI*, DNA was purified, and the DNA fragment was circularized with T4 ligase. This was introduced into *Escherichia coli* and then plasmid was collected from *Escherichia coli* that acquired ampicillin resistance. The plasmid thus collected contains genomic sequences adjacent to the right border (RB) of T-DNA and most regions of T-DNA in the msh mutant.

The nucleotide sequence of 5610 bp genomic DNA adjacent to the RB was determined, and the obtained nucleotide sequence was analyzed by the GENSCAN algorithm (<http://CCR-081.mit.edu/GENSCAN.html>) to predict the presence of a gene. As a result, it was found that the transcription of the gene which is closest to RB is initiated from a nucleotide at position 882 from RB, and the gene was designated MSH.

Example 3. Isolation of MSH cDNA

From the whole plant of the msh mutant and the wild type *Arabidopsis thaliana*, RNA was extracted, from which mRNA was purified using oligotex dT30 (Nippon Roche).

Using this as a template, a cDNA library was created using the lambda ZAPII cDNA library synthesis kit (Stratagene) according to a method recommended by Stratagene. cDNA libraries of these msh transformant and the wild type *Arabidopsis thaliana* were screened using the MSH gene obtained in Example 2 as a probe. Even screening of about 300,000 clones from the cDNA library derived from the wild type did not yield cDNA corresponding to the MSH gene, suggesting that the expression of the MSH gene in the wild type *Arabidopsis thaliana* is very weak or it is expressed in specific cell alone.

On the other hand, by screening about 20,000 clones of the cDNA library derived from the msh transformant, 31 positive clones were obtained, of which a clone designated M6 was used for the subsequent analysis. The nucleotide sequence of the M6 clone was determined, and the sequence is shown in SEQ ID NO: 1 of the sequence listing. The amino acid sequence corresponding to the nucleotide sequence is shown in SEQ ID NO: 2.

The full-length sequence of this coding region is contained in the MSH gene, and it was revealed that M6 was cDNA corresponding to the MSH gene. From the analysis of the nucleotide sequence of the cDNA, it was found that a protein encoded by the MSH gene has a significant homology with the homeodomain protein, and that the sequence corresponding to the third  $\alpha$  helix in the homeodomain conserved between homeodomain proteins is most conserved in the protein encoded by the MSH gene. Furthermore, the amino acid sequence of the protein encoded by the MSH gene had the highest homology with the sequence of WUSCHEL among the homeodomain proteins.

However, even when compared to WUSCHEL having the highest homology with MSH among those reported proteins, the ratio of identical amino acids in the homeodomain is 42% and about 20% in the overall protein, so that it cannot be concluded whether it has a similar function to

WUSCHEL based on the sequence. Of the homeodomain proteins, next to WUSCHEL the proteins of the KN1 type had the second highest homology with MSH protein. When the proteins were compared to MSH within the homeodomain for the homology, the ratio of identical amino acids was 20% or less. During the course of cloning, a cDNA clone of a sequence that had an identity of 86% within the homeodomain and 40% in the entire region with MSH cDNA was isolated and was designated M8. The nucleotide sequence is shown in SEQ ID NO: 3 of the sequence listing, and the corresponding amino acid sequence is shown in SEQ ID NO: 4.

Example 4. Formation of adventitious shoots by overexpression of MSH cDNA

As was predicted in Example 2, it was analyzed whether the overexpression of the MSH gene causes the formation of adventitious shoots. From a binary vector pBE2113GUS (Plant Cell Physiology, 37: 49-59, 1996, obtained from NIAR) the GUS gene was removed by treating with restriction enzymes BamHI/SacI. Instead, a coding region of MSHM6 cDNA was amplified by PCR using primer #170 (5'-GAAGATCTCATCATGTCCTCCTCAAAC-3') (SEQ ID NO: 5) and primer #172 (5'-CGGAGCTCTAAATAAGATAATAGATTGCGC-3') (SEQ ID NO: 6), and then a DNA fragment treated with restriction enzymes BglIII/SacI was integrated. By this procedure, the MSH cDNA inserted into the binary vector is placed under the control of an artificial promoter derived from CaMV35S promoter. The plasmid was designated pBE2113MSH.

pBE2113GUS and pBE2113MSH were introduced into the wild type *Arabidopsis thaliana* callus via *Agrobacterium*. Transformed cells were selected using kanamycin-resistance as an index. The transformed callus into which pBE2113GUS had been introduced required cytokinin at the time of adventitious shoot formation, whereas the callus transformed with pBE2113MSH was capable of regenerating adventitious shoots regardless of the

presence of cytokinin. In the presence of cytokinin, calluses transformed with either plasmid regenerated adventitious shoots, whereas the callus transformed with pBE2113MSH regenerated adventitious shoots more rapidly than the callus transformed with pBE2113GUS. Furthermore, *Arabidopsis thaliana* callus that overexpresses CKI1 cDNA, a previously reported sensor histidine of the two-component system can form adventitious shoots in the absence of cytokinin, but the number of adventitious shoots formed was greater in the callus that is overexpressing MSH cDNA.

On the other hand, pBE2113MSH was introduced into germ cells of *Arabidopsis thaliana* using the *Agrobacterium*-mediated vacuum infiltration method (Bechtold et al., C.R. Acad. Sci. Paris, Life Sciences, 316: 1194-1199, 1993; Takashi Araki, Shokubutu Saibo Kogaku Series 4 (Plant Cell Engineering Series 4), Experimental Protocol for Model Plants, pp. 109-113, 1996), and young plants into which the gene was introduced were selected using kanamycin resistance as an index. The transformant *Arabidopsis thaliana* thus obtained was found to have more branching than the wild type strain.

Furthermore, concerning the function of the protein encoded by M8 cDNA that encodes a protein homologous to MSH cDNA obtained in Example 3, a fusion protein of the protein encoded by M8 cDNA and GUS was analyzed by allowing its overexpression in *Arabidopsis thaliana* plant. The coding region of M8 cDNA was amplified by PCR using primer #224 (5'-GCTCTAGAACAATGGCTTCTTCGAATAGACAC-3') (SEQ ID NO: 7) and primer #225 (5'-TCCCCCGGGCTGATCAGATAGTACGAGGCTCC-3') (SEQ ID NO: 8), and then treated with restriction enzymes XbaI/SmaI, and the resulting gene fragment was inserted in between the XbaI/SmaI recognition sites of pBE2113GUS.

The binary vector pBE2113M8GUS thus obtained was introduced into germ cells of *Arabidopsis thaliana* using

the *Agrobacterium*-mediated vacuum infiltration method, and young plants into which the gene had been introduced were selected using kanamycin resistance as an index. The *Arabidopsis thaliana* transformant thus obtained was  
5 found to have more branching as does the above *Arabidopsis thaliana* mutant that overexpressed the above MSHM6 cDNA.

#### Industrial Applicability

10 The above data suggested that the gene MSH obtained by activation tagging from *Arabidopsis thaliana* is a homeobox gene involved in adventitious shoot formation, and is estimated to encode a transcription factor of the gene involved in adventitious shoot formation. Based on  
15 the results of overexpression of MSH cDNA under the control of the 35S promoter, it was suggested that MSH promotes adventitious shoot formation regardless of the presence of cytokinin, and, besides, that it is also involved in branching of plants.

20 From the foregoing, it is now possible to control adventitious shoot formation and branching formation from plants or plant cells by regulating the expression of the MSH gene.



CLAIMS

1. A gene encoding a protein that has the amino acid sequence as set forth in SEQ ID NO: 2, that is involved in differentiation, and that has a homeodomain-like sequence.

2. A gene encoding a protein that has an amino acid sequence modified by the addition or deletion of one or a plurality of amino acids and/or replacement with other amino acids in the amino acid sequence as set forth in SEQ ID NO: 2, that is involved in differentiation, and that has a homeodomain-like sequence.

3. A gene that hybridizes to the nucleic acid having the nucleotide sequence as set forth in SEQ ID NO: 1 or a portion thereof under a stringent condition, and that encodes a protein that is involved in differentiation and that has a homeodomain-like sequence.

4. A gene encoding a protein that has the amino acid sequence as set forth in SEQ ID NO: 4, that is involved in differentiation, and that has a homeodomain-like sequence.

5. A gene encoding a protein that has an amino acid sequence modified by the addition or deletion of one or a plurality of amino acids and/or replacement with other amino acids in the amino acid sequence as set forth in SEQ ID NO: 4, that is involved in differentiation, and that has a homeodomain-like sequence.

6. A gene that hybridizes to the nucleic acid having the nucleotide sequence as set forth in SEQ ID NO: 3 or a portion thereof under a stringent condition, and that encodes a protein that is involved in differentiation and that has a homeodomain-like sequence.

7. The gene according to any one of the claims 1 to 6 wherein said protein is a protein having an ability of inducing adventitious shoots.

8. The gene according to any one of the claims 1 to 6 wherein said protein is a protein having an ability of inducing branching.

9. A vector comprising the gene according to any one of the claims 1 to 8.

10. A host transformed with the vector according to claim 9.

5 11. A protein encoded by the gene according to any one of the claims 1 to 8.

12. A method for producing a protein that is involved in differentiation and that has a homeodomain-like sequence, said method comprising culturing or  
10 growing the host according to claim 10 and then harvesting said protein from said host.

13. The method for producing a protein according to claim 12, wherein said protein has an ability of inducing adventitious shoots.

15 14. The method for producing a protein according to claim 12, wherein said protein has an ability of inducing branching.

15. A plant or a plant cell into which the gene according to any one of the claims 1 to 8 has been  
20 introduced.

16. A method for inducing differentiation from a plant or a plant cell said method comprising introducing the gene according to any one of the claims 1 to 8 into a plant or a plant cell and then driving the expression of  
25 said gene.

17. A method for inducing adventitious shoot formation from a plant or a plant cell said method comprising introducing the gene according to any one of the claims 1 to 8 into a plant or a plant cell and then  
30 driving the expression of said gene.

18. A method for inducing branching of a plant said method comprising introducing the gene according to any one of the claims 1 to 8 into a plant and then driving the expression of said gene.

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I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

HOMEBOX GENE ENCODING A PROTEIN INVOLVED  
IN DIFFERENTIATION

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### Prior Foreign Application(s)

外国での先行出願 11-207995(Pat. Appln.)	Japan
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